The Effect of Vector with Higher Control System in hbFGF Leaky Expression in *Escherichia coli*

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The *hbfgf* (human basic fibroblast growth factor) gene cloning in a pET-3a expression vector, in spite of having the appropriate results in the amount of hbFGF hormone expression, after a while it leads to the leaky expression. Our attempts in order to find its several causes and solve the above problem resulted in sub-cloning the mentioned gene in the pET-22b vector. The obtained results demonstrated the acceptable amount of the gene production. Moreover there was no leaky expression in the host cell before induction. It can be concluded that it is due to the existence of an extra repression system for preventing the *hbfgf* gene to be transcribed before the induction. The extra repressor is a *lacO* sequence in the down stream of the T7 promoter and a *lacI* gene to encode *lac* repressor.

Keywords: Leaky Expression, *E.coli*, hbFGF, pET-22b

Introduction

However, the expression of T7RNA polymerase exists in some pET expression vectors (Novagen) [1], it leads to express considerably amount of foreign protein before induction with IPTG (leaky expression) [2]. The actual reason of this problem has not been known until now. It may be due to the few quantity of lactose in the medium [3]. The mentioned problem should be solve, since it causes to decrees considerably the rate of host cell and result in the reduction of the product [4]. One of the simplest solutions is adding glucose (50mg/ml) in to the medium in order to control the leaky expression [5]. However, it is not certainly definite solution for this problem. A more reliable approach is using the pET vectors with higher control system for foreign gene expression. In the previous research [6], when we cloned *hbfgf* gene in the expression vector pET-3a, the leaky expression was observed after a while. The influences of various media and medium (M9-Glu, M9-Gly and LB-Glu) composition on the target expression were evaluated [5]. In this study the leaky expression is reduced by sub-cloning the *hbfgf* gene in the pET-22b vector with an extra repression system. The pET-22b vector combines very tight transcriptional control with high levels of gene expression. Therefore, its ability to transcribe target gene in uninduced cells is decreased.

Materials & Methods

E.coli strains BL21 (DE3) pLysS (F^-, *omp*T, *hsd*$S_{B,}$ gal, dcm), and pET-22b expression vector were purchased from Novagene company. All chemicals were from standard commercial sources, unless noted.

The *hbfgf* gene was isolated from pET-1005 (cDNA-*hbfgf* gene inserted in pET-3a plasmid) with two restriction enzyme, *Pst*I and *Xba*I, [4] and ligated into the mentioned enzyme sites of plasmid pET-22b. The new construct was named pET-1006 (Figure 1).

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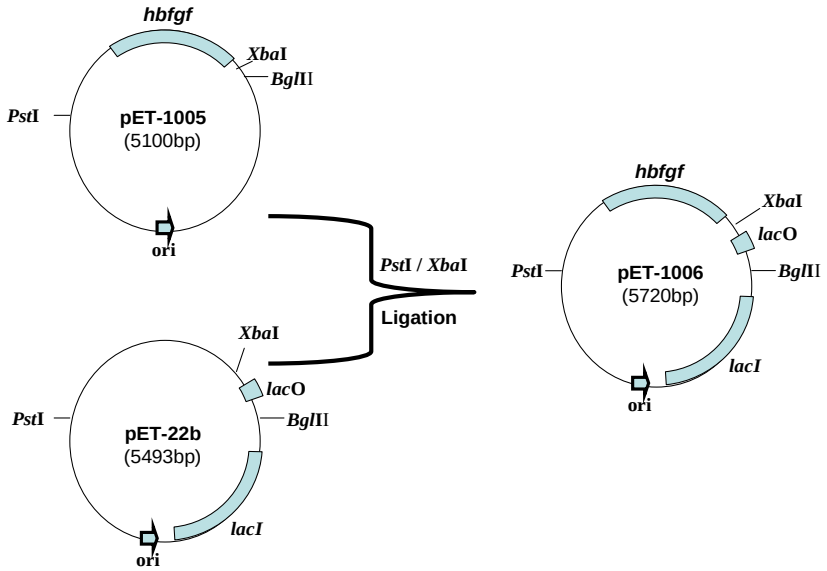

Figure 1: Construction of plasmid pET-1006: a *Xba*I-*Pst*I fragment bearing the *hbfgf* gene was rele the pET-1005 and then inserted in pET-22b vector.

To confirm right cloning, the *hbfgf* gene digested with *Eco*RV. Three expected segments were 200bp, 1900bp and 3600bp. For more confirmation the pET-1006 construct was digested with *Bgl*II and *Pst*I and expected two segments 3920bp and 1800bp. Seed stocks derived from early long phase cells (A_{600} = 0.1-0.5) frozen in 15% glycerol at -80°C, inoculated the cultures. In first stage seed stocks were transformed to Top10 host cells. The pET-1006 was transformed to the BL21 (DE3) pLysS (F^-, *omp*T, *hsd*S_B, gal, dcm) for expression. Shake flask (0.2-0.4% inoculum) including 5ml of LB medium and the appropriate antibiotic (Ampicilin, 50µg/ml) were inoculated by BL21-pET-1006. Cells were grown in 37°C and either induced with IPTG or left uninduced (as negative control). After five hours, the cells were collected by centrifugation and lysed by sonication (4 times, 30 sec). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% polyacrylamide gels were applied to analyze the induced as well as uninduced cell lysates based on the method of Laemmli [7].

Western blot was used in order to confirm hbFGF protein band. For western immunoblot analysis, the proteins which were separated on the gel were transformed onto a nitrocellulose membrane (0.2 m pore size, *schlecher & Schuell*). The first antibody (rabbit anti-hbFGF anti serum, SIGMA) and the second Antibody (horse radish peroxidase conjugated goat-anti-rabbit Ab, SIGMA), were added and left at room temperature for 2 hours. The immunoreaction was visualized using 0.5 mg/ml diaminobenzidine HCL and 0.01% H2O2. For quantitative measurement of hbFGF, an ELISA kit (R&D, USA) was used which includes a standard hbFGF for plotting a standard curve.

Results

Sub-cloning of hbfgf gene in pET-22b:

The new pET-1006 construct which is a derivative of pET-1005(cDNA-*hbfgf* gene inserted in pET-3a plasmid) was constructed in this study. The *hbfgf* gene was isolated from pET-1005 by two restriction enzyme (*Pst*I and *Xba*I). The mentioned gene inserted to the expression vector, pET-22b, to result in a new construct was termed pET-1006. To confirm right cloning, the pET-1006 digested with *Eco*RV and *Pst*I/*Bgl*II (Figure- 2).

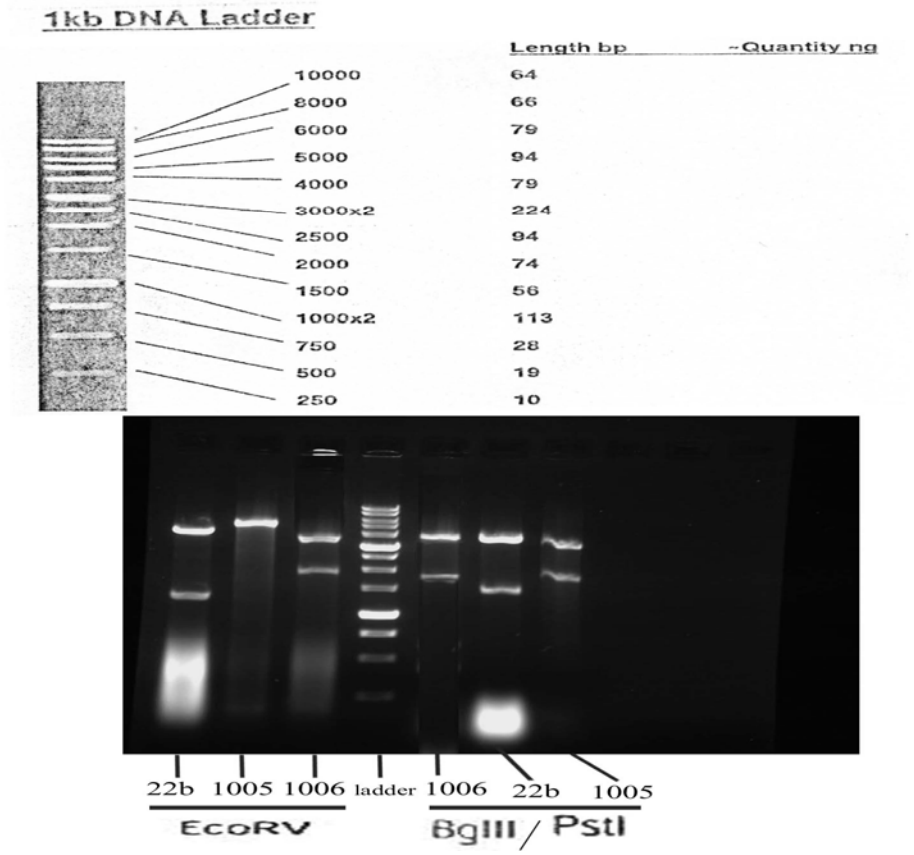

Figure 2: Digestion of pET-1005, pET-1006 & pET-22b with *Eco*RV and *Pst*I / *Bgl*II restriction enzymes to confirm right cloning for pET-1006.

Expression & Detection:

Cells containing pET-1006 were grown and induced by IPTG. The result from SDS-PAGE showed (Figure-3) which there is no leaky expression for uninduced cells (negative control) of pET-1006.

The result of western blotting analysis shows that there are intense bands corresponding to standard hbFGF for pET-1005 and pET-1006 constructs (Figure-4). We have also determined the amount of protein by ELISA kit. The results showed that the production of pET-1005 and pET-1006 were 0.9 mg/ml and 0.8 mg/ml respectively.

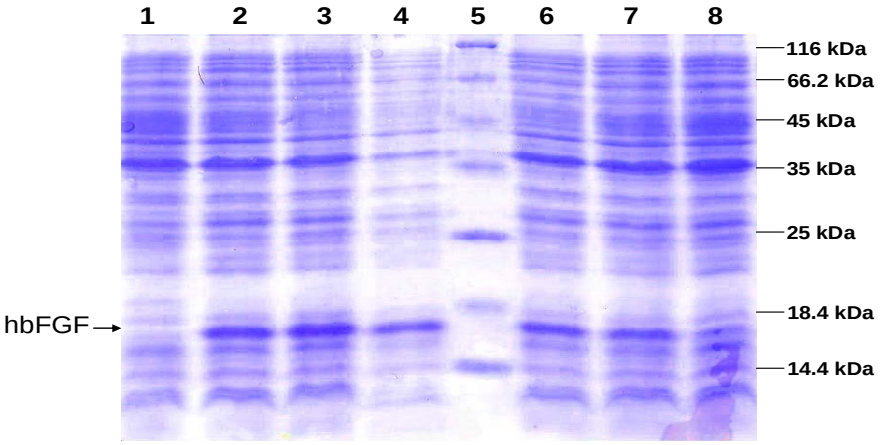

Figure 3: SDS-PAGE (15%); Lane 1: Negative control (cells without plasmid); Lanes 2 & 3: pET-1005; Lane 4: pET-1005 (uninduced cells); Lane 5: Protein size marker; Lanes 6 & 7: pET-1006; Lane 8: pET-1006 (uninduced cells).

Discussion

It is always likely that some difficulties be occurred attempting to express a heterologous gene in *E.coli* or other hosts [8]. Although the new expression vectors have been used extensively to produce high level of recombinant protein in *E.coli*, problem of leaky expression do sometimes arise. [9].

To minimize the leaky expression problem, it is usual to use a vector in which the cloned gene is under the control of a very tight regular promoter. In previous research we enhanced the amount of production of hbFGF hormone in a phage-T7 System (pET-3a) [5, 6]. However, after a while it was found that the T7 promoter controls the leaky expression in uninduced *E.coli* /BL21 (DE3) pLysS cell that is expressing hbFGF [6]. Providing a source of T7 RNA polymerase from the *lac*UV5 promoter in λDE3 lysogens can causes the expression induction [10]. The induction with IPTG (an analog of lactose) causes the T7RNA polymerase gene to be expressed and transcribes the recombinant DNA sequence [2]. However, even the absence of IPTG (inducer), there is some expression of T7 RNA polymerase [1].
To overcome this problem, the influences of various media and medium (M9-Glu, M9-Gly and LB-Glu) composition on the target expression were evaluated [5]. One approach is to use vectors that is termed T7*lac* promoter [11, 12].

In this study the *hbfgf* gene was inserted in the pET-22b expression vector (with T7 lac promoter) which was selected. As a result the new pET-1006 construct was prepared. This plasmid contains a *lac* operator sequence just downstream of the T7 promoter. They also carry the natural promoter and coding sequence for the *lac* repressor (*lacI*) [12]. The *lacI* repressor gene is transcribed and the *lacI* repressor binds to the lac operator (*lacO*) sequence, preventing expression of the cloned gene [9]. Therefore, the pET-22b vector offers extremely tight control of expression of cloned genes. As shown in Figure-3, 4, the leaky expression decreased considerably in pET-1006 construct (*hbfgf* gene inserted in pET-22b plasmid) in comparison with pET-1005.

Here, it can be concluded that the leaky expression may have different reasons. It can cause the negative effect on expression of target protein. Recently, it has been shown that derepression of the *lac* operon in the absence of inducer may be part of a general cellular response to nutrient limitation [3].

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